B1 IDS 1654

In the United States Patent & Trademark Office

In re Application of:
Canne, L., *et al.*

Serial No.: **09/987,655**

Filed on: November 15, 2001

For: Solid Phase Native Chemical Ligation of UnProtected or N-Terminal Cysteine Protected Peptides in Aqueous Solution

Examiner: Anish, Gupta

Art Unit: 1654

Atty Dkt. No.: 03504.284B

Response to Notice of Drawing Inconsistency with Specification

Hon. Commissioner for Patents
P.O. Box 1450
Alexandri, VA 22313-1450

Mail Stop Issue Fee

Sir:

Further to the Notice of Drawing Inconsistency with Specification issued on August 31, 2005 with respect to the above-identified patent application, the time for responding to which runs to September 30, 2005, Applicants herewith respectfully respond as follows:

1. The Notice of Drawing Inconsistency with Specification issued on August 31, 2005 states that "Figures 32A-H are contained in the Drawings but not listed in Brief Description of the Drawings in the Specification." A copy of the Notice is enclosed.

2. It is believed that the Notice is in error.

3. The Brief Description of the Drawings in the originally filed Specification contained erroneous references to Figures 32A-H (reference to Figures 30A-H had been intended). No drawings comprising Figures 32A-H were ever filed in this application.

4. The Specification references to Figure 32A-H were corrected (so as to reference Figures 30A-H) in a Preliminary Amendment filed on November 15, 2001. A copy of the Preliminary Amendment is enclosed.

5. A Notice Regarding Drawings issued on July 11, 2005 advising Applicants to conform Figure 30, sheets A and B, so that Figure 30 would more clearly identify sub-figures A-H.

6. Applicants responded to such Notice on July 20, 2005. A copy of Applicants' response and the United States Patent and Trademark Office Notice are enclosed.

7. It is therefore respectfully submitted that the specification's Brief Descriptions of the Drawings now fully corresponds to the submitted Drawings (particularly with respect to figures 30A-H).

8. Accordingly no further response is believed to be required.

The commissioner is invited to contact the undersigned in the event that any further action is required.

Date: 9/6/2005

Respectfully Submitted,

Berenato, White & Stavish, LLC
6550 Rock Spring Drive, Suite 240
Bethesda, MD 20817
Telephone: (301) 896-0600
Facsimile: (301) 896-0607

Jeffrey I. Auerbach
Reg. No. 32,680
Attorney for Assignee

UNITED STATES PATENT AND TRADEMARK OFFICE

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/987,655	11/15/2001	Lynne Canne	03504.284B	7459

7590 08/31/2005

LINIAK BERENATO LONGACRE & WHITE, LLC
SUITE 240
6550 ROCK SPRING DRIVE
BETHESDA, MD 20817

EXAMINER	
GUPTA, ANISH	
ART UNIT	**PAPER NUMBER**
1654	

DATE MAILED: 08/31/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND
DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE

NOTICE OF DRAWING INCONSISTENCY WITH SPECIFICATION

The drawings filed 1/28/02 have been received. However, an inconsistency exists between the drawings and the Brief Description of the Drawings in the specification.

Figures _____ are listed in the Brief Description of the Drawings in the specification but not contained in the Drawings.

Figures 32A-H are contained in the Drawings but not listed in the Brief Description of the Drawings in the specification.

Applicant is required to correct the above-noted inconsistency within a time period of **ONE MONTH or THIRTY (30) DAYS, whichever is longer,** from the mailing date of this Notice, or within the time remaining in the time period set forth in the Notice of Allowability (Form PTOL-37) to file corrected drawings, whichever is longer. **NO EXTENSION OF THIS TIME PERIOD MAY BE GRANTED UNDER EITHER 37 CFR 1.136 (a) OR (b)**

Failure to correct the above noted inconsistency will result in **abandonment** of the application.

The file will be held in the Publishing Division to await the correction of the inconsistency.

Return Corrected Drawings/Specification to:
Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450

Alexandria, VA 22313-1450

Office of Patent Publication/Publishing Division
Customer Service: 703-308-6789
1-888-786-0101

FORM PTO-1631 (REV. 10-03)

In the United States Patent & Trademark Office

In re Application of:
Canne, L., *et al.*

Serial No.: **09/987,655**

Filed on: November 15, 2001

For: Solid Phase Native Chemical Ligation of UnProtected or N-Terminal Cysteine Protected Peptides in Aqueous Solution

Examiner: Anish, Gupta

Art Unit: 1654

Atty Dkt. No.: 03504.284B

Response to Notice Regarding Drawings

Hon. Commissioner for Patents
P.O. Box 1450
Alexandri, VA 22313-1450

Sir:

In Response to the Notice Regarding Drawings issued on July 11, 2005 with respect to the above-identified patent application, the time for responding to which runs to September 11, 2005, Applicants herewith submit:

1. Notice Regarding Drawings issued on July 11, 2005
2. Amendment requesting entry of Replacement Sheets (2 sheets) for Figure 30
3. Replacement Sheets (2 sheets) for Figure 30
4. Annotated Marked Up Replacement Sheets (2 sheets) for Figure 30

Respectfully Submitted,

Date: 20-July-2005

Berenato, White & Stavish, LLC
6550 Rock Spring Drive, Suite 240
Bethesda, MD 20817
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Jeffrey I. Auerbach
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Attorney for Assignee

JUL 12 2005

UNITED STATES PATENT AND TRADEMARK OFFICE

UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND
DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMES APPLICANT	ATTORNEY DOCKET NUMBER
09/987,655	01/28/2002	LYNNE CANNE	03504.284B

LINIAK BERENATO LONGACRE & WHITE, LLC
SUITE 240
6550 ROCK SPRING DRIVE
BETHESDA, MD 20817

Examiner
GUPTA, ANISH

Art Unit: 1654

Paper Number

Date Mailed:7/11/2005

Notice Regarding Drawings

Corrected drawings for the above-identified application, received in the USPTO on are still not acceptable for the reason(s) identified on the attached PTO-948. Applicant is given one opportunity to correct the informalities within a two-month time period from the mailing date of this Notice. **THIS TIME PERIOD IS NOT EXTENDABLE UNDER EITHER 37 CFR 1.136(a) OR 1.136(b). Failure to take corrective action within the set period will result in abandonment of the application.**

ATTACHMENT: PTO-948 Notice of Draftsperson's Patent Review

RETURN CORRECTED DRAWINGS TO:
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Clifton Randolph
Office of Patent Publication,
Publishing Division
703-305-0333 ext-128

Form PTO-948 (Rev. 06/03)
Application No. 09/987,655

U.S. DEPARTMENT OF COMMERCE
U.S. Patent and Trademark Office

NOTICE OF DRAFTSPERSON'S PATENT DRAWING REVIEW

The drawing(s) filed (insert date) 11/28/02 are:

A. ___ approved by the Draftsperson under 37 CFR 1.84 or 1.152.

B. ✓ objected to by the Draftsperson under 37 CFR 1.84 or 1.152 for the reasons indicated below. Corrected drawings are required.

1. DRAWINGS. 37 CFR 1.84(a): Acceptable categories of drawings: Black ink or Color (3 sets required).
___ Color drawings are not acceptable until petition is granted. Fig(s)___
___ Pencil and non black ink not permitted. Fig(s)___

2. PHOTOGRAPHS. 37 CFR 1.84(b)
___ One (1) full-tone set is required. Fig(s)___
___ Photographs may not be mounted. 37 CFR 1.84(e)
___ Photographs must meet paper size requirements of 37 CFR 1.84(f). Fig(s)___
___ Poor quality (half-tone). Fig(s)___

3. TYPE OF PAPER. 37 CFR 1.84(e)
___ Paper not flexible, strong, white, and durable. Fig(s)___
___ Erasures, alterations, overwritings. interlineations, folds, copy machine marks not accepted. Fig(s)___

4. SIZE OF PAPER. 37 CFR 1.84(f): Acceptable sizes:
21.0 cm by 29.7 cm (DIN size A4) or
21.6 cm by 27.9 cm (8 1/2x 11 inches)
___ All drawing sheets not the same size. Sheet(s)___
___ Drawings sheets not an acceptable size. Fig(s)___

5. MARGINS. 37 CFR 1.84(g): Acceptable margins: Top 2.5 cm Left 2.5 cm Right 1.5 cm Bottom 1.0 cm
___ Margins not acceptable. Fig(s)___
_____Top (T) _____ Left (L)
_____Right (R) _____ Bottom (B)

6. VIEWS. 37 CFR 1.84(h)
REMINDER: Specification may require revision to correspond to drawing changes, e.g., if Fig. 1 is changed to Fig. 1A, Fig 1B and Fig. 1C, etc., the specification, at the Brief Description of the Drawings, must likewise be changed.
___Views not labeled separately or properly. Fig(s)___

7. SECTIONAL VIEWS. 37 CFR 1.84(h)(3)
____ Sectional designation should be noted with Arabic or Roman numbers. Fig(s)___

8. ARRANGEMENT OF VIEWS. 37 CFR 1.84(i)
___ Words do not appear on a horizontal, left-to-right fashion when page is either upright or turned so that the top becomes the right side, except for graphs. Fig(s)___

9. SCALE. 37 CFR 1.84(k)
___ Scale not large enough to show mechanism without crowding when drawing is reduced in size to two-thirds in reproduction. Fig(s)___

10. CHARACTER OF LINES, NUMBERS, & LETTERS. 37 CFR 1.84(l)
___ Lines, numbers & letters not uniformly thick and well defined, clean, durable, and black (poor line quality). Fig(s)___

11. SHADING. 37 CFR 1.84(m)
___ Solid black areas pale. Fig(s) ___
___ Solid black shading not permitted. Fig(s)___

12. NUMBERS, LETTERS, & REFERENCE CHARACTERS. 37 CFR 1.84(p)
___ Numbers and reference characters not plain and legible. Fig(s)___
___ Figure legends are poor. Fig(s)___
___ Numbers and reference characters not oriented in the same direction as the view. 37 CFR 1.84(p)(1) Fig(s)___
___ English alphabet not used. 37 CFR 1.84(p)(2) Fig(s)___
___ Numbers, letters and reference characters must be at least 32 cm (1/8 inch) in height. 37 CFR 1.84(p)(3). Fig(s)___

13. LEAD LINES. 37 CFR 1.84(q)
___ Lead lines missing. Fig(s)___

14. NUMBERING OF SHEETS OF DRAWINGS. 37 CFR 1.84(t)
___ Sheets not numbered consecutively, and in Arabic numbers beginning with number 1. Sheet(s)___

15. NUMBERING OF VIEWS. 37 CFR 1.84(u)
✓ Views not numbered consecutively, and in Arabic numerals, beginning with number 1. Fig(s) 30

16. DESIGN DRAWINGS. 37 CFR 1.152
___ Surface shading shown not appropriate. Fig(s)___
___ Solid black surface shading is not permitted except when used to represent the color black as well as color contrast. Fig(s)___

COMMENTS: Renumber as Fig 30A and Fig 30B

Reviewer QBR

Date 7/11/05

1 26 59
GLLDLKSMIEKVTGKNALTNYGFYG**CYCGWGGRGTPKDGTDWCCWAHDHCYGRLEEKG**C
NIRTQSYKYRFAWGV VTCEPGPFCHVNL**CACDRKLVYCLKRNLRSYNPQYQYFPNILCS**
88 118

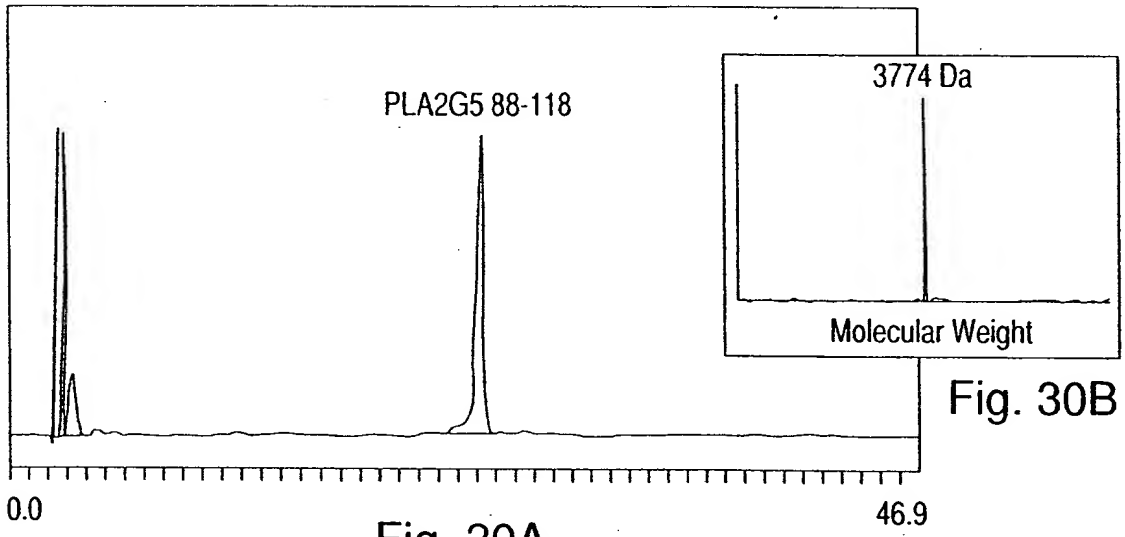

Fig. 30B

Fig. 30A

1 26 59
GLLDLKSMIEKVTGKNALTNYGFYG**CYCGWGGRGTPKDGTDWCCWAHDHCYGRLEEKG**C
NIRTQSYKYRFAWGV VTCEPGPFCHVNL**CACDRKLVYCLKRNLRSYNPQYQYFPNILCS**
88 118

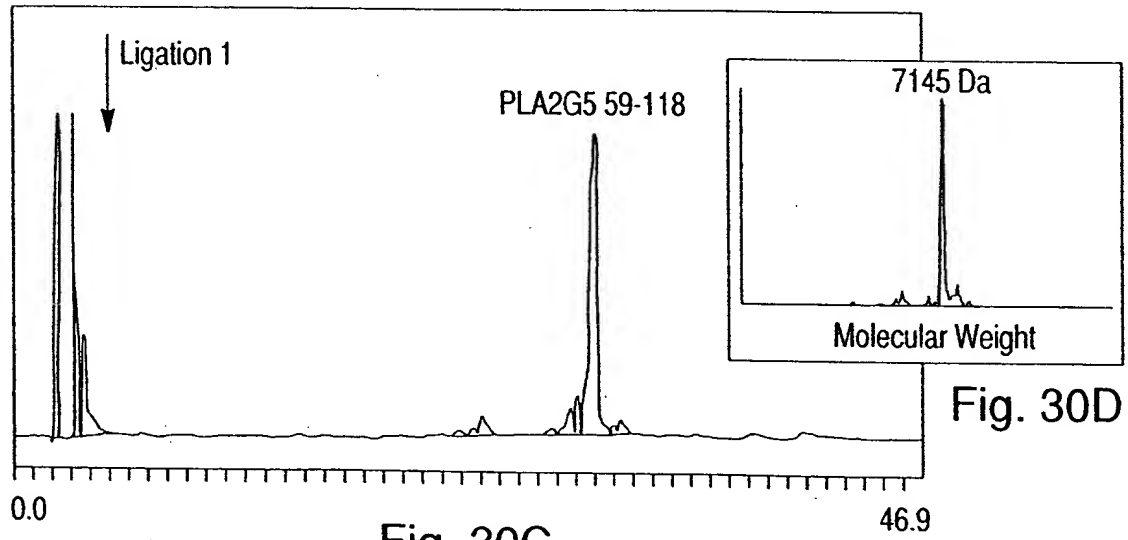

Fig. 30D

Fig. 30C

1 26 59
GLLDLKSMIEKVTGKNALTNYGFYG**CYCGWGGRGTPKDGTDWCCWAHDHCYGRLEEKG**C
NIRTQSYKYRFAWGV VTCEPGPFCHVNL**CACDRKLVYCLKRNLRSYNPQYQYFPNILCS**
88 118

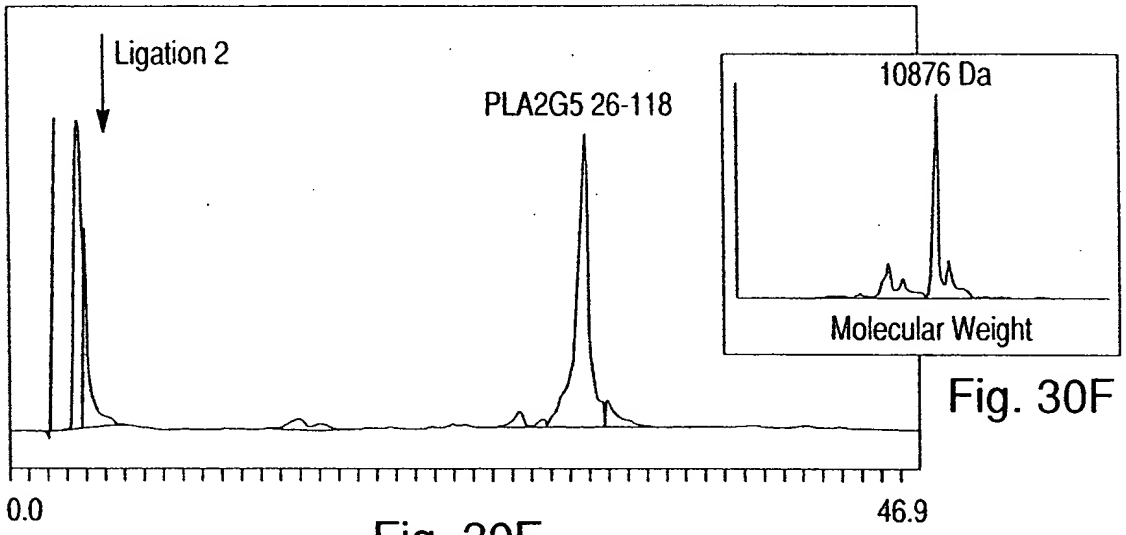

Fig. 30F

Fig. 30E

1 26 59
GLLDLKSMIEKVTGKNALTNYGFYG**CYCGWGGRGTPKDGTDWCCWAHDHCYGRLEEKG**C
NIRTQSYKYRFAWGV VTCEPGPFCHVNL**CACDRKLVYCLKRNLRSYNPQYQYFPNILCS**
88 118

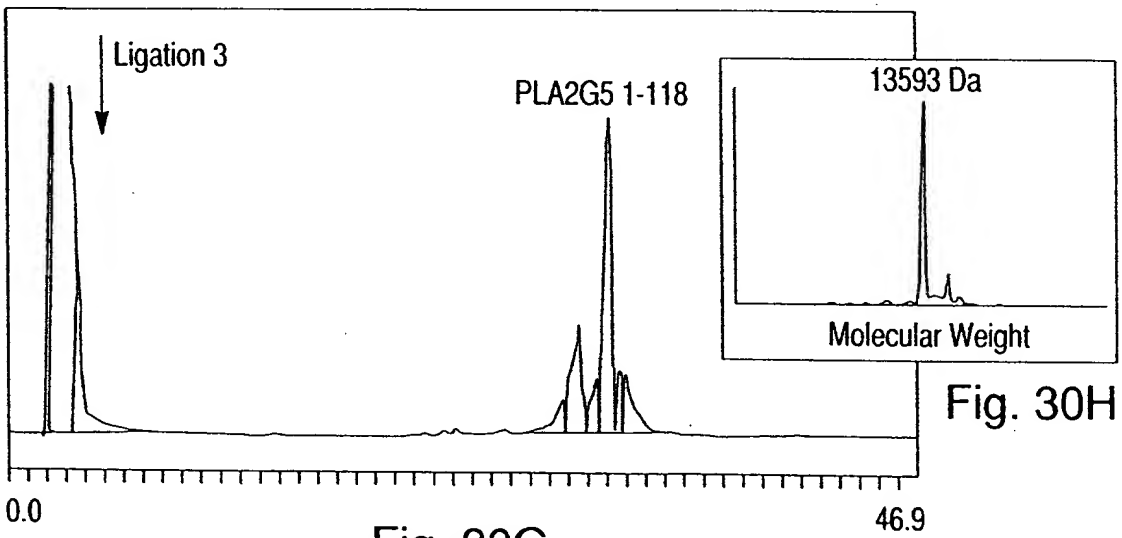

Fig. 30H

Fig. 30G

In the United States Patent & Trademark Office

In re Application of: **Canne, L., *et al.***	Examiner:
Serial No.: To Be Provided	Art Unit:
Filed on: Herewith	
(Divisional of U.S. Patent Appln. Ser. No.: 09/097,094 Filed on June 12, 1998)	
For: Solid Phase Chemical Ligation of UnProtected or N-Terminal Cysteine Protected Peptides in Aqueous Solution	Atty Dkt. No.: 03504.284B

PRELIMINARY AMENDMENT

Hon. Director of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Prior to commencing the examination of the above-described patent application, Applicants respectfully request entry and consideration of the following Preliminary Amendments.

Amendments

In the Specification:

Please replace pages 1, 14, 15, 32, 34, 36, 38, and 42 and Figure 30 of the Specification with the enclosed replacement pages (Appendix C). The specific nature of the changes made to these pages is shown in Appendix A.

Please incorporate the enclosed SEQUENCE LISTINGS (Appendix C) after page 49 of the Specification.

In the Claims:

Please cancel claims 1-26 and 28.

Please amend claim 27 to read as follows:

27. **[Amended]** An apparatus for producing assembled polypeptides, comprising:

(a) a solid support, having bound thereto a first partially or completely unprotected peptide having an N-terminus and a thioacid or a thioester of the formula –COSR at its C-terminus, wherein said partially or completely unprotected peptide is bound to said solid support via a linker; wherein said linker comprises a cleavable moiety and said partially or completely unprotected first peptide segment is bound to said linker at said N-terminus, and wherein R is a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof;

(b) a set of second partially or completely unprotected peptides, each comprising a thioester or a thioacid at its C- terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said second peptide segment is capable of selectively ligating to the C-terminus of said solid phase-bound first peptide to form a solid phase-bound peptide comprising a thioacid at its C-terminus; and

(c) one or more sets of different partially or completely unprotected peptides, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each of the members of each set have the same number of amino acids. --

Please add the following new claims:

-- 29. **[New]** The apparatus of claim 27, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.

30. **[New]** The apparatus of claim 27, wherein the C-terminus of said first partially or completely unprotected peptide comprises said thioester of formula COSR.

31. **[New]** The apparatus of claim 27, wherein the C-terminus of said second partially or completely unprotected peptide comprises a thioacid.

32. **[New]** The apparatus of claim 27, wherein the C-terminus of said second partially or completely unprotected peptide comprises said thioester of formula COSR.

33. **[New]** The apparatus of claim 27, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.

34. **[New]** The apparatus of claim 27, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.

35. **[New]** The apparatus of claim 27, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.

36. **[New]** The apparatus of claim 27, wherein said solid phase is a bead resin.

37. **[New]** The apparatus of claim 27, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.

38. **[New]** The apparatus of claim 27, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.

39. **[New]** The apparatus of claim 27, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.

40. **[New]** The apparatus of claim 27, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.

41. **[New]** An apparatus for preparing assembled polypeptides comprising:

a) a solid phase support having bound thereto a partially or completely unprotected first peptide segment comprising an N-terminus and a C-terminus, wherein said peptide is bound to said support via a cleavable linkage between C-terminus and said support, and wherein said N-terminus is a cysteine residue.

b) a set of second partially or completely unprotected peptides, each comprising a thioester of formula COSR or a thioacid at its C-terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said solid phase-bound first peptide segment is capable of selectively ligating to the C-terminus of said second peptide to form a solid phase-bound peptide comprising a cysteine at its N-terminus; wherein R is a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof; and

c) one or more sets of different partially or completely unprotected peptide segments, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each of the members of each set have the same number of amino acids. --

42. **[New]** The apparatus of claim 41, wherein said set of second unprotected peptide segments is comprised of peptides having the same length, but different amino acid sequences.

43. **[New]** The apparatus of claim 41, wherein said set of second unprotected peptides consists essentially of identical peptides.

44. **[New]** The apparatus of claim 41, wherein said one or more sets of different unprotected peptides (c) comprise at least one set of peptides having the same length but different amino acid sequences.

45. **[New]** The apparatus of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.

46. **[New]** The apparatus of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises said thioester of formula COSR.

47. **[New]** The apparatus of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises a thioacid.

48. **[New]** The apparatus of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises said thioester of formula COSR.

49. **[New]** The apparatus of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.

50. **[New]** The apparatus of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.

51. **[New]** The apparatus of claim 41, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.

52. **[New]** The apparatus of claim 41, wherein said solid phase is a bead resin.

53. **[New]** The apparatus of claim 41, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.

54. **[New]** The apparatus of claim 41, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.

55. **[New]** The apparatus of claim 41, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.

56. **[New]** The apparatus of claim 41, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure. --

Remarks

Status

Applicants have cancelled claims 1-26 and 28. Accordingly, Claim 27 is pending and new Claims 29-56 have been added.

Applicants have amended the claims to more clearly describe Applicants invention. The present invention pertains to an apparatus for preparing assembled polypeptides. Support for the recitation of "partially or completely unprotected peptide" can be found at page 5, lines 3-5. Applicants have additionally amended the claims to recite the presence of "a thioacid or thioester of the formula -COSR." Support for this recitation can be found on page 6, lines 17-18 and page 7, lines 1-2. The claims have additionally been amended to recite the formula –COSR, wherein R is " a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof." Support for this recitation can be found on page 18, lines 8-11.

The claims have additionally been amended to recite a second peptide segment that is "capable of selectively ligating to the C-terminus of said solid phase-bound first peptide to form a solid phase-bound peptide comprising a thioacid at its C-terminus." Support for this recitation can be found on page 6, lines 18-24.

Applicants have additionally added new claims to recite a partially or completely unprotected peptide comprising "a thioester of the formula COSR" or "a thioacid." Support for this recitation can be found on page 6, lines 15-20 and page 18, lines 7-13.

Applicants have additionally added claims to recite a polypeptide of "20 to 1000 amino acids in length." Support for this recitation can be found on page 16, line 30. Support for the recitation of the solid phase being a bead resin can be found on page 17, line 20. Support for the recitation of a peptide segment of "5 to 99 amino acids in length" can be found on page 17, line 1. Support for the recitation of a peptide segment prepared by "solid phase synthesis" can be found on page 17, line 5. Support for the recitation of a "last peptide segment derived from recombinant DNA expression" can be found on page 17, line 7. Support for the recitation of a peptide segment with "an unnatural backbone structure" can be found on page 17, line 3. The remaining amendments to the claims have been made solely to remedy minor informalities or to address typographical or formatting errors.

The specification has been amended to recite that the present application is a divisional of United States Patent Application Serial no. 09/097,094. In addition, pages 14 and 15 have been amended to conform to the Figures submitted with the original application. As will be recognized, original Figures 26, 28, and 29 do not contain Figure A and Figure B components. The text of the specification has accordingly been amended to conform to the original Figures. On page 15, the specification referred to Figure 32(A-H). As will again be recognized, this is a typographical error; the text is referring to original Figure 30. Since original Figure 30 does not contain A-H components, the Figure has been amended to add reference to such components.

No new matter has been introduced by any of the requested amendments. Applicants respectfully submit that the present application is in condition for Examination, and earnestly solicit early notice of favorable action. The Examiner is invited to contact the undersigned regarding any issue in this case.

Respectfully Submitted,

Date: 11/15/2001

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Bethesda, MD 20817
Telephone: (301) 896-0600
Facsimile: (301) 896-0607

Jeffrey I. Auerbach
Reg. No. 32,680
Attorney for Applicants

Appendix A: The Nature of the Requested Amendments

To facilitate the Examiner's review of the patentability of the present invention, Applicant has reproduced below the specific nature of the requested amendments.

In the Specification:

Page 1 has been amended as follows:

SOLID PHASE NATIVE CHEMICAL LIGATION
OF UNPROTECTED OR N-TERMINAL CYSTEINE PROTECTED PEPTIDES
IN AQUEOUS SOLUTION

CROSS-REFERENCE TO RELATED APPLICA TIONS

This application **is a continuation-in part of, and** claims the benefit of, U.S. Provisional Application No. 60/049,553, filed June 13, 1997**, herein incorporated by reference, and is a divisional application of, and claims the benefit of, U.S. Patent Application Serial No. 09/097,094, herein incorporated by reference**.

Background

Existing methods for, the chemical synthesis of proteins include stepwise solid phase synthesis, and fragment condensation either in solution or on solid phase. The classic stepwise solid phase synthesis of Merrifield involves covalently linking an amino acid corresponding to the carboxy-terminal amino acid of the desired peptide chain to a solid support and extending the polypeptide chain toward the amino end by stepwise coupling of activated amino acid derivatives having activated carboxyl groups. After completion of the assembly of the fully protected solid phase bound peptide chain, the peptide-solid phase covalent attachment is cleaved by suitable chemistry and the protecting groups removed to give the product polypeptide.

Some disadvantages of the stepwise solid phase synthesis method include: incomplete reaction at the coupling and deprotection steps in each cycle results in formation of solid-phase bound by products. Similarly, side reactions due to imperfections in the chemistry, and or impurities present in the reagents/protected amino acids, all lead to a multiplicity of solid phase bound products at each step of the chain assembly and to the formation of complex product mixtures in the final product. Thus, the longer the peptide chain, the more challenging it is to obtain high-purity well-defined

products. Due to the production of complex mixtures, the stepwise solid phase synthesis approach has size limitations. In general, well-defined polypeptides of 100 amino acid residues or more are not routinely prepared via stepwise solid phase synthesis. Synthesis of proteins and large polypeptides by this route is a time-consuming and laborious task. --

Page 14 has been amended as follows:

-- group of the N-terminal cysteine. Steps 2 and 3 can be repeated, as indicated by the arrow marked 4, for additional peptide segments. Also, a cleavable linker for purposes of monitoring the coupling and ligating reactions can be added between the "handle" and the "resin."

FIG. 22 is a reaction scheme for solid phase sequential ligation in the C- to N-terminal direction of PLA2G5.

FIG. 23 is a reaction scheme for synthesizing a Cam ester derivative for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 24 is a reaction scheme for synthesizing the C-terminal peptide segment for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 25A, B, and **C** is a diagram of a scheme for synthesizing an assembled polypeptide via bidirectional solid phase sequential ligation of two or more peptide segments.

FIG 26[A and B] are HPLC chromatographs following the solid phase solid phase native chemical ligation of 3 peptide segments in the N- to C- terminal direction, resulting in the assembled peptide, C5a 1-74.

FIG. 27 is a reaction scheme for synthesis of a C-terminal peptide segment for use in the solid phase native chemical ligations described herein, using a CAM ester cleavable handle to remove the synthesized peptide segment from the solid phase.

FIG. 28**[A and B]** are HPLC chromatographs and reconstructed ESI MS of the assembled peptide resulting from solid phase sequential ligation of 3 peptide segments: peptide segment 1 **[(SEQ ID NO: 2)]**(CADRKNILA) **(amino acids 19-27; SEQ ID NO:1)**, peptide segment 2 **[(SEQ ID NO:3)]** (CYGRLEEKG) **(amino acids 10-18; SEQ ID NO:1)** and peptide segment 3 **[(SEQ ID NO:4)]** (ALTKYGFYG) **(amino acids 1-9; SEQ ID NO:1)** on solid phase in the C- to N-terminal direction, using Fmoc protecting groups.

FIG. 29[A and B] are an HPLC chromatograph and ESI MS**[, respectively,]** of the final

ligation product, i.e. the first ligation product ligated to the third peptide segment (ALTKYGFYG) **(amino acids 1-9; SEQ ID NO:1)**, resulting from solid phase sequential ligation of 3 peptide segments in the C- to N-terminal direction, using ACM as the protecting group.

FIG. 30A-H are HPLC chromatographs and reconstructed ESI MS of the steps of synthesizing Phospholipase A2 Group 5, a 118 residue protein, using solid phase sequential native chemical ligation of four peptide segments in the C- to N-terminal direction. The first peptide segment is PLA2G5 88-118; the second is PLA2G5 59-87, the third is PLA2G5 26-58,

Page 15 has been amended as follows:

-- and the fourth is PLA2G5 1-25. **FIG 30[32]A and B** are an HPLC chromatograph and reconstructed ESI MS of the first peptide segment, respectively. **FIG 30[32]C and D** are an HPLC chromatograph and reconstructed ESI MS, respectively, of the ligation product of the first and second peptide segments (PLA2G5 59-118). **FIG 30[32]E and F** are **[an]** an HPLC chromatograph and reconstructed ESI MS, respectively, of PLA2G5 26-118, the ligation product of PLA2G5 59-118 and PLA2G5 26-58 (the third peptide segment). **FIG 30[32]G and H** are HPLC chromatograph and reconstructed ESI MS, respectively, ofPLA2G5 1-118, the assembled polypeptide.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Terminology

Amino acids: Amino acids include the 20 genetically coded amino acids, rare or unusual amino acids that are found in nature, and any of the non-naturally occurring and modified amino acids.

Aqueous solution: solutions containing water, including up to 8M urea in water, up to 6M guanidine- HCl in water, up to 60% acetonitrile in water.

Assembled Peptide: the final product of a solid phase sequential or bidirectional ligation, after cleavage of the cleavable handle, The assembled peptide comprises at least two separate peptide segments sequentially ligated on a solid phase. The assembled peptide mayor may not have biological activity.

Cleavable Handle: A cleavable moiety that is capable of being selectively cleaved to release the assembled peptide from the solid phase. The cleavable handle must be capable of resisting cleavage under conditions suitable for coupling, activating, deprotecting, ligating, washing, and other

steps involved in the formation of an assembled peptide. The cleavable handle must also be stable to conditions used to produce the first peptide segment that is capable of being bound to a solid phase, including, for example, stepwise solid phase peptide synthesis. The cleavable handle preferably is located directly adjacent to the first peptide segment such that upon cleavage of the cleavable handle, the desired assembled peptide is released from the solid phase. The cleavable handle may be selected from any of the variety of cleavable handles used by those in the field. See, e.g., L. Canne et al., Tetrahedron Letters, 38(19):3361-3364 (1997); Ball et al., J. Pept. Sci, 1:288-294 (1995); Funakoshi et al, PNAS USA, 88:6981-6985 (1991); --

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Page 32 has been amended solely to introduce the phrase –(SEQ ID NO:2) -- at the last line of the depicted reaction schemes.

Table 1

Solid Phase Sequential Ligations: N- to C- Terminal

3-Random Peptide Segment Model System

Lev-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS) (1)

\+ *Resin-PCL-ONH2*

↓ 1. pH 4.6, 6M GuHCl, 0.1 M acetate

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS) (1)

↓ 2. pH 4.6, 6M GuHCl, 0.1 M acetate, 50 mM BrAcOH

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-*COSAc* (1)

↓ + *H*-CGFRVREFGDNTA-COS) (2)

3. pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

↓ CGFRVREF-GDNTA-COS) (1+2)

4. pH 4.6, 6M GuHCl, 0.1M acetate, 50mM BrAcOH

Resin- *PCL-oxime- MSC* -LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

CGFRVREF-GDNTA-*COSAc* (1+2)

\+ *H*-CADPSEEWVQKYVSDLELSA-*OH* (3)

↓ 5. pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol

Resin- *PCL-oxime- MSC* –LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

CGFRVREF-GDNTACADPSEEWVQKYVSDLELSA-*OH* (1+2+3)

↓ 6. pH 14, 6M GuHCl, 0.1M phosphate, 200mM hydrazine

H-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHGCGFRVREF-GDNTACADPSEEWVQKYVSDLELSA-*OH* (1+2+3) **(SEQ ID NO:2)**

PCL= photocleavable linker

Page 34 has been amended as follows:

reaction mixture is left standing at room temperature overnight. The next morning, the resin is washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample of resin are removed for monitoring by MALDI MS analysis.

The assembled peptide is removed from the solid phase via base cleavage of the cleavable handle from the remaining resin as outlined above only on a larger scale followed by purification by HPLC or desalting on PD-10 column and lyophilization.

Example 4: Solid Phase Native Chemical Ligation of C5a(1-74) (74aa) in the N- to C- Terminal Direction.

This example describes solid phase sequential native chemical ligation in the N- to C-terminal direction of C5a, Complement Factor 5A. The sequence of C5a is: **[(SEQ ID NO. ?)]**

TLQKKIEEEIAAKYKJSVVKKCCYDGACVNNDETCEQRAARISLGPKCIKAFTECCVVAS

QLRANISHKDMQLGR **(SEQ ID NO:3)**.

This peptide is prepared using solid phase sequential native ligation of 3 peptide segments: C5a(I-20), C5a(21-46), and C5a(47-74). The procedures used to synthesize C5a by solid phase ligations are identical to those described in the solid phase sequential native ligation of MIF (See Example 5).

Example 5: Solid Phase Sequential Native Chemical Ligation of MIF(1-115) (115 aa) in the N-Terminal to C- Terminal Direction.

The sequence of MIF(1-115) is **[(SEQ.ID.NO.)]**:

MPMFIVNTNVPRASVPDGFLSELTQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCAL

CSLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAASVGWNNSTFA **(SEQ ID NO:4)**

This peptide is prepared using solid phase sequential native ligation of 3 peptide segments: MIF(1-59) **(amino acid 1-59, SEQ ID NO:4)** MIF(60-80) **(amino acid 60-80, SEQ ID NO:4)** and MIF(81-115) **(amino acid 81-115, SEQ ID NO:4)**. See FIG. 16-20.

Page 36 has been amended as follows:

-- Example 6: Solid Phase Native Chemical. Ligation of Phospholipase A2, group 5(1-118) (118aa) in the C- to N-terminal Direction.

The sequence of Phospholipase A2, group 5 (PLA2G5) is: **[(SEQ ID NO:):]**
GLLDLKSMIEKVTGKNALTNYGFYGCYCGWGGRGTPKDGTDWCCWAHDHCYGRLEE
KGCNIRTQSYKYRFAWGVVTCEPGPFCHVNLCACDRKLVYCLKRNLRSYNPQYQYFPN
ILCS **(SEQ ID NO:5)**.

This peptide is prepared using solid phase sequential native ligation of 4 peptide segments: PLA2G5 (1-25), PLA2G5 (26-58), PLA2G5(59-87) and PLA2G5 (88-118). The procedures used to synthesize PLA2G5 by solid phase ligations are identical to those used for synthesizing the random sequence using ACM protection of the N-terminal Cys residues of the middle segments, as described in Example 9. See FIG. 22 for the reaction scheme. The Cam ester derivative is synthesized and incorporated into the C-terminal peptide segment according to the diagrams in FIG. 23, 24/FIG. 27. The assembled polypeptide, PLA2G5 (1-118), was folded and assayed for biological activity. It had the full activity of a recombinantly expressed PLA2G5.

Example 7: Preparation of Modified C-terminal Peptide Segment (on-resin CAM linker synthesis) (FIG. 27)
The commerical resin of choice (MBHA, any Boc-AA-OCH2-Pam resin) is swelled in DMF
-TFA (1 min x 2) (not necessary if working with MBHA resin)
-DMF flow wash (30 sec x 2)
-addition of activated Boc-Lys(Fmoc)-OH (HBTU/DIEA activation), check for completion of reaction after 10-15 minutes by ninhydrin test
-DMF flow wash (30 sec x 2)
-TFA (1 min x 2) --

Page 38 has been amended as follows:

-- Example 8: Solid Phase Native Chemical Ligation of Random Peptide Segments in the C- to N-terminal Direction using Fmoc protection (See FIG. 28)

The following procedures can be used for solid phase ligations in the C- to N-terminal direction, as diagramed in Table 2. By example, a random peptide of **[(SEQ ID NO: ?)]**: ALTKYGFYGCYGRLEEKGCADRKNILA **(SEQ ID NO:1)** can be ligated in three peptide segments (from C- to N-terminal direction): segment 1= CADRKNILA **(amino acids 19-27; SEQ ID NO:1)**; segment 2 = CYGRLEEKG **(amino acids 10-18; SEQ ID NO:1)**; and segment 3 = ALTKYGFYG **(amino acids 1-9; SEQ ID NO:1)**.

The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 m1 x 5) and drained. The modified C-terminal peptide segment (first peptide segment) is dissolved in 6MGu•HCL, 0.1M Na Acetate, pH 4.6 (5 mM first peptide segment) and added to the resin and is left standing at room temperature overnight. The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample is removed for base cleavage and is treated with 8M urea, 0.1M NaPi, pH 7, treated for 2 minutes with 0.25N NaOH in the same 8M urea buffer (resulting pH~14), washed with an equal amount of 0.25N HCl in the same 8M urea buffer (resulting pH~2), and the combined eluants treated with TCEP prior to injection on HPLC.

In preparation for addition of the next segment, the resin is washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5) and drained. The second peptide segment (Fmoc-Cys-peptide-COSR) is dissolved in 6M Gu•HCl, 0.1M NaPi, pH 7.0, 0.5% thiophenol (to at least 10 mM to 50 mM second peptide segment) and added to the resin. **[the]** **The** mixture is left standing at room temperature overnight. The resin is washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5), water (1 ml x 5), DMF (1 ml x 5), and the Fmoc protecting group removed by treating with two aliquots of 20% piperidine in DMF (5 min each). The resin is then washed with DMF (1 ml x 5), water (1 --

Page 42 has been amended as follows: -- Table 2

Polymer-Supported Ligations

C- to N- Terminal Direction

Fmoc Protection

H-CADRKNILA-*CAM-Lys(Levulinic acid)-NH2* (1)

+ Resin- ONH_2

↓ 1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H -CADRKNILA -*CAM- Lys-oxime*-Resin (1)

+ *Fmoc*-CYGRLEEKG-*COSR* (2)

↓ 2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

Fmoc-CYGRLEEKGCADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

↓ 3. 20% piperidine/DMF

H-CYGRLEEKGCADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

+ *H*-ALTKYGFYG-*COSR* (3)

↓ 4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-ALTKYGFYGCYGRLEEKGCADRKNILA-*CAM-Lys-oxime-Resin* (1+2+3)

↓ 5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYGCYGRLEEKGCADRKNILA-*OH* **(SEQ ID NO:1)** --

Page 43 has been amended to read: -- Table 3

Polymer-Supported Ligations

C- to N- Terminal Direction

ACM Protection

H-CADRKNILA-*CAM-Lys(Levulinic acid)-NH2* (1)

+ Resin- ONH_2

↓ 1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H -CADRKNILA -*CAM-Lys-oxime*-Resin (1)

+ *H*-**C***(ACM)***YGRLEEKG***-COSR* (2)

↓ 2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-**C***(ACM)***YGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

↓ 3. a. mercury(II)acetate in 3% Aq. AcOH
b. 20% mercaptoethanol in pH 7.5, 6M Gu•HCl, 0.1 M phosphate

H-**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

+ *H*-ALTKYGFYG-*COSR* (3)

↓ 4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H- ALTKYGFYG**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2+3)

↓ 5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYG**CYGRLEEKG**CADRKNILA-*OH* **(SEQ ID NO:1)** --

Figure 30 has been amended to include Figure sub-legends A-H.

In the Claims:

-- 27. **[Amended]** An apparatus for producing assembled polypeptides, comprising:

(a) [an unprotected peptide segment, comprising] a solid support, having bound thereto a first **partially or completely** unprotected peptide having **an N-terminus and a thioacid or** a thioester **of the formula –COSR** at its C-terminus [and a cleavable linker at its N-terminus], wherein said **partially or completely** unprotected peptide is bound to said solid support via a linker; **wherein said linker comprises a cleavable moiety and said partially or completely unprotected first peptide segment is bound to said linker at said N-terminus, and wherein R is a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof;**

(b) a set of second **partially or completely** unprotected peptides, each comprising **a thioester or** a thioacid at **its** [their C- termini] **C-terminus** and a cysteine residue at **its** [their N-termini] **N-terminus**; **wherein the N-terminal cysteine of said second peptide segment is capable of selectively ligating to the C-terminus of said solid phase-bound first peptide to form a solid phase-bound peptide comprising a thioacid at its C-terminus;** and

(c) one or more sets of different **partially or completely** unprotected peptides, each comprising a **thioester or a** thioacid at **its** [their C-termini] **C-terminus** and a cysteine residue at **its** [their N-termini] **N-terminus, wherein each of the members of each set have the same number of amino acids**. --

29. **[New]** The apparatus of claim 27, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.

30. **[New]** The apparatus of claim 27, wherein the C-terminus of said first partially or completely unprotected peptide comprises said thioester of formula COSR.

31. **[New]** The apparatus of claim 27, wherein the C-terminus of said second partially or completely unprotected peptide comprises a thioacid.

32. **[New]** The apparatus of claim 27, wherein the C-terminus of said second partially or completely unprotected peptide comprises said thioester of formula COSR.

33. **[New]** The apparatus of claim 27, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.

34. **[New]** The apparatus of claim 27, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.

35. **[New]** The apparatus of claim 27, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.

36. **[New]** The apparatus of claim 27, wherein said solid phase is a bead resin.

37. **[New]** The apparatus of claim 27, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.

38. **[New]** The apparatus of claim 27, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.

39. **[New]** The apparatus of claim 27, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.

40. **[New]** The apparatus of claim 27, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.

41. **[New]** An apparatus for preparing assembled polypeptides comprising:

a) a solid phase support having bound thereto a partially or completely unprotected first peptide segment comprising an N-terminus and a C-terminus, wherein said peptide is bound to said support via a cleavable linkage between C-terminus and said support, and wherein said N-terminus is a cysteine residue.

b) a set of second partially or completely unprotected peptides, each comprising a thioester of formula COSR or a thioacid at its C-terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said solid phase-bound first peptide segment is capable of selectively ligating to the C-terminus of said second peptide to form a solid phase-bound peptide comprising a cysteine at its N-terminus; wherein R is a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof; and

c) one or more sets of different partially or completely unprotected peptide segments, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each

of the members of each set have the same number of amino acids. --

42. **[New]** The apparatus of claim 41, wherein said set of second unprotected peptide segments is comprised of peptides having the same length, but different amino acid sequences.

43. **[New]** The apparatus of claim 41, wherein said set of second unprotected peptides consists essentially of identical peptides.

44. **[New]** The apparatus of claim 41, wherein said one or more sets of different unprotected peptides (c) comprise at least one set of peptides having the same length but different amino acid sequences.

45. **[New]** The apparatus of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.

46. **[New]** The apparatus of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises said thioester of formula COSR.

47. **[New]** The apparatus of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises a thioacid.

48. **[New]** The apparatus of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises said thioester of formula COSR.

49. **[New]** The apparatus of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.

50. **[New]** The apparatus of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.

51. **[New]** The apparatus of claim 41, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.

52. **[New]** The apparatus of claim 41, wherein said solid phase is a bead resin.

53. **[New]** The apparatus of claim 41, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.

54. **[New]** The apparatus of claim 41, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.

57. **[New]** The apparatus of claim 41, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.

58. **[New]** The apparatus of claim 41, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.

Appendix B: The Pending Claims

To facilitate the Examiner's review of the patentability of the present invention, Applicant has reproduced below the presently pending claims.

27. **[Amended]** An apparatus for producing assembled polypeptides, comprising:

(a) a solid support, having bound thereto a first partially or completely unprotected peptide having an N-terminus and a thioacid or a thioester of the formula –COSR at its C-terminus, wherein said partially or completely unprotected peptide is bound to said solid support via a linker; wherein said linker comprises a cleavable moiety and said partially or completely unprotected first peptide segment is bound to said linker at said N-terminus, and wherein R is a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof;

(b) a set of second partially or completely unprotected peptides, each comprising a thioester or a thioacid at its C- terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said second peptide segment is capable of selectively ligating to the C-terminus of said solid phase-bound first peptide to form a solid phase-bound peptide comprising a thioacid at its C-terminus; and

(c) one or more sets of different partially or completely unprotected peptides, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each of the members of each set have the same number of amino acids.

29. **[New]** The apparatus of claim 27, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.

30. **[New]** The apparatus of claim 27, wherein the C-terminus of said first partially or completely unprotected peptide comprises said thioester of formula COSR.

31. **[New]** The apparatus of claim 27, wherein the C-terminus of said second partially or completely unprotected peptide comprises a thioacid.

32. **[New]** The apparatus of claim 27, wherein the C-terminus of said second partially or completely unprotected peptide comprises said thioester of formula COSR.

33. **[New]** The apparatus of claim 27, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.

34. **[New]** The apparatus of claim 27, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.

35. **[New]** The apparatus of claim 27, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.

36. **[New]** The apparatus of claim 27, wherein said solid phase is a bead resin.

37. **[New]** The apparatus of claim 27, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.

38. **[New]** The apparatus of claim 27, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.

39. **[New]** The apparatus of claim 27, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.

40. **[New]** The apparatus of claim 27, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.

41. **[New]** An apparatus for preparing assembled polypeptides comprising:

a) a solid phase support having bound thereto a partially or completely unprotected first peptide segment comprising an N-terminus and a C-terminus, wherein said peptide is bound to said support via a cleavable linkage between C-terminus and said support, and wherein said N-terminus is a cysteine residue.

b) a set of second partially or completely unprotected peptides, each comprising a thioester of formula COSR or a thioacid at its C-terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said solid phase-bound first peptide segment is capable of selectively ligating to the C-terminus of said second peptide to form a solid phase-bound peptide comprising a cysteine at its N-terminus; wherein R is a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof; and

c) one or more sets of different partially or completely unprotected peptide segments, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each of the members of each set have the same number of amino acids. --

42. **[New]** The apparatus of claim 41, wherein said set of second unprotected peptide segments is comprised of peptides having the same length, but different amino acid sequences.

43. **[New]** The apparatus of claim 41, wherein said set of second unprotected peptides consists essentially of identical peptides.

44. **[New]** The apparatus of claim 41, wherein said one or more sets of different unprotected peptides (c) comprise at least one set of peptides having the same length but different amino acid sequences.

45. **[New]** The apparatus of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.

46. **[New]** The apparatus of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises said thioester of formula COSR.

47. **[New]** The apparatus of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises a thioacid.

48. **[New]** The apparatus of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises said thioester of formula COSR.

49. **[New]** The apparatus of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.

50. **[New]** The apparatus of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.

51. **[New]** The apparatus of claim 41, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.

52. **[New]** The apparatus of claim 41, wherein said solid phase is a bead resin.

53. **[New]** The apparatus of claim 41, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.

54. **[New]** The apparatus of claim 41, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.

59. **[New]** The apparatus of claim 41, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.

60. **[New]** The apparatus of claim 41, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.

APPENDIX C

APPENDIX C

SOLID PHASE NATIVE CHEMICAL LIGATION OF UNPROTECTED OR N-TERMINAL CYSTEINE PROTECTED PEPTIDES IN AQUEOUS SOLUTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in part of, and claims the benefit of, U.S. Provisional Application No. 60/049,553, filed June 13, 1997, herein incorporated by reference, and is a divisional application of, and claims the benefit of, U.S. Patent Application Serial No. 09/097,094, herein incorporated by reference.

Background

Existing methods for, the chemical synthesis of proteins include stepwise solid phase synthesis, and fragment condensation either in solution or on solid phase. The classic stepwise solid phase synthesis of Merrifield involves covalently linking an amino acid corresponding to the carboxy-terminal amino acid of the desired peptide chain to a solid support and extending the polypeptide chain toward the amino end by stepwise coupling of activated amino acid derivatives having activated carboxyl groups. After completion of the assembly of the fully protected solid phase bound peptide chain, the peptide-solid phase covalent attachment is cleaved by suitable chemistry and the protecting groups removed to give the product polypeptide.

Some disadvantages of the stepwise solid phase synthesis method include: incomplete reaction at the coupling and deprotection steps in each cycle results in formation of solid-phase bound by products. Similarly, side reactions due to imperfections in the chemistry, and or impurities present in the reagents/protected amino acids, all lead to a multiplicity of solid phase bound products at each step of the chain assembly and to the formation of complex product mixtures in the final product. Thus, the longer the peptide chain, the more challenging it is to obtain high-purity well-defined products. Due to the production of complex mixtures, the stepwise solid phase synthesis approach has size limitations. In general, well-defined polypeptides of 100 amino acid residues or more are not routinely prepared via stepwise solid phase synthesis. Synthesis of proteins and large polypeptides by this route is a time-consuming and laborious task.

group of the N-terminal cysteine. Steps 2 and 3 can be repeated, as indicated by the arrow marked 4, for additional peptide segments. Also, a cleavable linker for purposes of monitoring the coupling and ligating reactions can be added between the "handle" and the "resin."

FIG. 22 is a reaction scheme for solid phase sequential ligation in the C- to N-terminal direction of PLA2G5.

FIG. 23 is a reaction scheme for synthesizing a Cam ester derivative for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 24 is a reaction scheme for synthesizing the C-terminal peptide segment for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 25A, B, and **C** is a diagram of a scheme for synthesizing an assembled polypeptide via bidirectional solid phase sequential ligation of two or more peptide segments.

FIG 26 are HPLC chromatographs following the solid phase solid phase native chemical ligation of 3 peptide segments in the N- to C- terminal direction, resulting in the assembled peptide, C5a 1-74.

FIG. 27 is a reaction scheme for synthesis of a C-terminal peptide segment for use in the solid phase native chemical ligations described herein, using a CAM ester cleavable handle to remove the synthesized peptide segment from the solid phase.

FIG. 28 are HPLC chromatographs and reconstructed ESI MS of the assembled peptide resulting from solid phase sequential ligation of 3 peptide segments: peptide segment 1 (CADRKNILA) (amino acids 19-27; SEQ ID NO:1), peptide segment 2 (CYGRLEEKG) (amino acids 10-18; SEQ ID NO:1) and peptide segment 3 (ALTKYGFYG) (amino acids 1-9; SEQ ID NO:1) on solid phase in the C- to N-terminal direction, using Fmoc protecting groups.

FIG. 29 are an HPLC chromatograph and ESI MS of the final ligation product, i.e. the first ligation product ligated to the third peptide segment (ALTKYGFYG) (amino acids 1-9; SEQ ID NO:1), resulting from solid phase sequential ligation of 3 peptide segments in the C- to N-terminal direction, using ACM as the protecting group.

FIG. 30A-H are HPLC chromatographs and reconstructed ESI MS of the steps of synthesizing Phospholipase A2 Group 5, a 118 residue protein, using solid phase sequential native chemical ligation of four peptide segments in the C- to N-terminal direction. The first peptide segment is PLA2G5 88-118; the second is PLA2G5 59-87, the third is PLA2G5 26-58,

and the fourth is PLA2G5 1-25. **FIG 30A and B** are an HPLC chromatograph and reconstructed ESI MS of the first peptide segment, respectively. **FIG 30C and D** are an HPLC chromatograph and reconstructed ESI MS, respectively, of the ligation product of the first and second peptide segments (PLA2G5 59-118). **FIG 30E and F** are an an HPLC chromatograph and reconstructed ESI MS, respectively, of PLA2G5 26-118, the ligation product of PLA2G5 59-118 and PLA2G5 26-58 (the third peptide segment). **FIG 30G and H** are HPLC chromatograph and reconstructed ESI MS, respectively, ofPLA2G5 1-118, the assembled polypeptide.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Terminology

Amino acids: Amino acids include the 20 genetically coded amino acids, rare or unusual amino acids that are found in nature, and any of the non-naturally occurring and modified amino acids.

Aqueous solution: solutions containing water, including up to 8M urea in water, up to 6M guanidine- HCl in water, up to 60% acetonitrile in water.

Assembled Peptide: the final product of a solid phase sequential or bidirectional ligation, after cleavage of the cleavable handle, The assembled peptide comprises at least two separate peptide segments sequentially ligated on a solid phase. The assembled peptide mayor may not have biological activity.

Cleavable Handle: A cleavable moiety that is capable of being selectively cleaved to release the assembled peptide from the solid phase. The cleavable handle must be capable of resisting cleavage under conditions suitable for coupling, activating, deprotecting, ligating, washing, and other steps involved in the formation of an assembled peptide. The cleavable handle must also be stable to conditions used to produce the first peptide segment that is capable of being bound to a solid phase, including, for example, stepwise solid phase peptide synthesis. The cleavable handle preferably is located directly adjacent to the first peptide segment such that upon cleavage of the cleavable handle, the desired assembled peptide is released from the solid phase. The cleavable handle may be selected from any of the variety of cleavable handles used by those in the field. See, e.g., L. Canne et al., Tetrahedron Letters, 38(19):3361-3364 (1997); Ball et al., J. Pept. Sci, 1:288-294 (1995); Funakoshi et al, PNAS USA, 88:6981-6985 (1991);

Table 1

Solid Phase Sequential Ligations: N- to C- Terminal

3-Random Peptide Segment Model System

Lev-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS) (1)

+ *Resin-PCL-ONH2*

↓ **1.** pH 4.6, 6M GuHCl, 0.1 M acetate

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS) (1)

↓ **2.** pH 4.6, 6M GuHCl, 0.1 M acetate, 50 mM BrAcOH

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-*COSAc* (1)

↓ + *H*-**CGFRVREFGDNTA**-COS) (2)

3. pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

↓ **CGFRVREF-GDNTA**-COS) (1+2)

4. pH 4.6, 6M GuHCl, 0.1M acetate, 50mM BrAcOH

Resin- *PCL-oxime- MSC* -LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

CGFRVREF-GDNTA-*COSAc* (1+2)

+ *H*-CADPSEEWVQKYVSDLELSA-*OH* (3)

↓ **5.** pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol

Resin- *PCL-oxime- MSC* –LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

CGFRVREF-GDNTACADPSEEWVQKYVSDLELSA-*OH* (1+2+3)

↓ **6.** pH 14, 6M GuHCl, 0.1M phosphate, 200mM hydrazine

H-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG**CGFRVREF-**

GDNTACADPSEEWVQKYVSDLELSA-*OH* (1+2+3) (SEQ ID NO:2)

PCL= photocleavable linker

reaction mixture is left standing at room temperature overnight. The next morning, the resin is washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample of resin are removed for monitoring by MALDI MS analysis.

The assembled peptide is removed from the solid phase via base cleavage of the cleavable handle from the remaining resin as outlined above only on a larger scale followed by purification by HPLC or desalting on PD-10 column and lyophilization.

Example 4: Solid Phase Native Chemical Ligation of C5a(1-74) (74aa) in the N- to C-Terminal Direction.

This example describes solid phase sequential native chemical ligation in the N- to C-terminal direction of C5a, Complement Factor 5A. The sequence of C5a is:
TLQKKIEEEIAAKYKJSVVKKCCYDGACVNNDETCEQRAARISLGPKCIKAFTECCVVAS QLRANISHKDMQLGR (SEQ ID NO:3).

This peptide is prepared using solid phase sequential native ligation of 3 peptide segments: C5a(I-20), C5a(21-46), and C5a(47-74). The procedures used to synthesize C5a by solid phase ligations are identical to those described in the solid phase sequential native ligation of MIF (See Example 5).

Example 5: Solid Phase Sequential Native Chemical Ligation of MIF(1-115) (115 aa) in the N- Terminal to C- Terminal Direction.

The sequence of MIF(1-115) is:
MPMFIVNTNVPRASVPDGFLSELTQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCAL CSLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAASVGWNNSTFA (SEQ ID NO:4)

This peptide is prepared using solid phase sequential native ligation of 3 peptide segments: MIF(1-59) (amino acid 1-59, SEQ ID NO:4) MIF(60-80) (amino acid 60-80, SEQ ID NO:4) and MIF(81-115) (amino acid 81-115, SEQ ID NO:4). **See FIG. 16-20.**

Example 6: Solid Phase Native Chemical. Ligation of Phospholipase A2, group 5(1-118) (118aa) in the C- to N-terminal Direction.

The sequence of Phospholipase A2, group 5 (PLA2G5) is:
GLLDLKSMIEKVTGKNALTNYGFYGCYCGWGGRGTPKDGTDWCCWAHDHCYGRLEE
KGCNIRTQSYKYRFAWGVVTCEPGPFCHVNLCACDRKLVYCLKRNLRSYNPQYQYFPN
ILCS (SEQ ID NO:5).

This peptide is prepared using solid phase sequential native ligation of 4 peptide segments: PLA2G5 (1-25), PLA2G5 (26-58), PLA2G5(59-87) and PLA2G5 (88-118). The procedures used to synthesize PLA2G5 by solid phase ligations are identical to those used for synthesizing the random sequence using ACM protection of the N-terminal Cys residues of the middle segments, as described in Example 9. **See FIG. 22** for the reaction scheme. The Cam ester derivative is synthesized and incorporated into the C-terminal peptide segment according to the diagrams in **FIG. 23, 24/FIG. 27**. The assembled polypeptide, PLA2G5 (1-118), was folded and assayed for biological activity. It had the full activity of a recombinantly expressed PLA2G5.

Example 7: Preparation of Modified C-terminal Peptide Segment (on-resin CAM linker synthesis) (FIG. 27)

The commerical resin of choice (MBHA, any Boc-AA-OCH2-Pam resin) is swelled in DMF
-TFA (1 min x 2) (not necessary if working with MBHA resin)
-DMF flow wash (30 sec x 2)
-addition of activated Boc-Lys(Fmoc)-OH (HBTU/DIEA activation), check for completion of reaction after 10-15 minutes by ninhydrin test
-DMF flow wash (30 sec x 2)
-TFA (1 min x 2) –

Example 8: Solid Phase Native Chemical Ligation of Random Peptide Segments in the C- to N-terminal Direction using Fmoc protection (See FIG. 28)

The following procedures can be used for solid phase ligations in the C- to N-terminal direction, as diagramed in Table 2. By example, a random peptide of: ALTKYGFYGCYGRLEEKGCADRKNILA (SEQ ID NO:1) can be ligated in three peptide segments (from C- to N-terminal direction): segment 1= CADRKNILA (amino acids 19-27; SEQ ID NO:1); segment 2 = CYGRLEEKG (amino acids 10-18; SEQ ID NO:1); and segment 3 = ALTKYGFYG (amino acids 1-9; SEQ ID NO:1).

The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. The modified C-terminal peptide segment (first peptide segment) is dissolved in 6MGu•HCL, 0.1M Na Acetate, pH 4.6 (5 mM first peptide segment) and added to the resin and is left standing at room temperature overnight. The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample is removed for base cleavage and is treated with 8M urea, 0.1M NaPi, pH 7, treated for 2 minutes with 0.25N NaOH in the same 8M urea buffer (resulting pH~14), washed with an equal amount of 0.25N HCl in the same 8M urea buffer (resulting pH~2), and the combined eluants treated with TCEP prior to injection on HPLC.

In preparation for addition of the next segment, the resin is washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5) and drained. The second peptide segment (Fmoc-Cys-peptide-COSR) is dissolved in 6M Gu•HCl, 0.1M NaPi, pH 7.0, 0.5% thiophenol (to at least 10 mM to 50 mM second peptide segment) and added to the resin. The mixture is left standing at room temperature overnight. The resin is washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5), water (1 ml x 5), DMF (1 ml x 5), and the Fmoc protecting group removed by treating with two aliquots of 20% piperidine in DMF (5 min each). The resin is then washed with DMF (1 ml x 5), water (1

Table 2

Polymer-Supported Ligations

C- to N- Terminal Direction

Fmoc Protection

H-CADRKNILA-*CAM-Lys(Levulinic acid)-NH2* (1)

+ Resin- ONH_2

↓ **1.** pH 4.6, 6M Gu•HCl, 0.1 acetate

H -CADRKNILA -*CAM- Lys-oxime*-Resin (1)

+ *Fmoc*-**CYGRLEEKG**-*COSR* (2)

↓ **2.** pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

Fmoc-**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

↓ **3.** 20% piperidine/DMF

H-**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

+ *H*-ALTKYGFYG-*COSR* (3)

↓ **4.** pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-ALTKYGFYG**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2+3)

↓ **5.** pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYG**CYGRLEEKG**CADRKNILA-*OH* (SEQ ID NO:1)

Polymer-Supported Ligations

C- to N- Terminal Direction

ACM Protection

H-CADRKNILA-*CAM-Lys(Levulinic acid)-NH2* (1)

+ Resin- ONH_2

↓ 1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H -CADRKNILA -*CAM-Lys-oxime*-Resin (1)

+ *H*-C*(ACM)***YGRLEEKG**-*COSR* (2)

↓ 2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-C*(ACM)***YGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

↓ 3. a. mercury(II)acetate in 3% Aq. AcOH
b. 20% mercaptoethanol in pH 7.5, 6M Gu•HCl, 0.1 M phosphate

H-**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

+ *H*-ALTKYGFYG-*COSR* (3)

↓ 4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H- ALTKYGFYG**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2+3)

↓ 5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYG**CYGRLEEKG**CADRKNILA-*OH* (SEQ ID NO:1)

SEQUENCE LISTING

<110> Canne, Lynne
Kent, Stephen B.H.
Simon, Reyna

<120> Solid Phase Native Chemical Ligation of Unprotected or N-Terminal Cysteine Protected Peptides in Aqueous Solution

<130> GRFN-023/01US

<140> 09/097,094
<141> 1998-06-12

<150> 60/049,553
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Lys Gly Cys Ala Asp Arg Lys Asn Ile Leu Ala
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Lys His Gly Cys Gly Phe Arg Val Arg Glu Phe Gly Asp Asn Thr Ala
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Cys Ala Asp Pro Ser Glu Glu Trp Val Gln Lys Tyr Val Ser Asp Leu
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Glu Leu Ser Ala
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<210> 3
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<213> Homo sapiens

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Val Lys Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
20 25 30

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Lys Cys Ile Lys
35 40 45

Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu Arg Ala Asn Ile
50 55 60

Ser His Lys Asp Met Gln Leu Gly Arg
65 70

<210> 4
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20 25 30

Lys Pro Pro Gln Tyr Ile Ala Val His Val Val Pro Asp Gln Leu Met
35 40 45

Ala Phe Gly Gly Ser Ser Glu Pro Cys Ala Leu Cys Ser Leu His Ser
50 55 60

```
Lys His Gly Cys Gly Phe Arg Val Arg Glu Phe Gly Asp Asn Thr Ala
         35                  40                  45

Cys Ala Asp Pro Ser Glu Glu Trp Val Gln Lys Tyr Val Ser Asp Leu
     50                  55                  60

Glu Leu Ser Ala
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<210> 3
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 Thr Leu Gln Lys Lys Ile Glu Glu Ile Ala Ala Lys Tyr Lys Ser Val
   1               5                  10                  15

Val Lys Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
             20                  25                  30

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Lys Cys Ile Lys
         35                  40                  45

Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu Arg Ala Asn Ile
     50                  55                  60

Ser His Lys Asp Met Gln Leu Gly Arg
 65                  70
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<210> 4
<211> 115
<212> PRT
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<400> 4

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Met Pro Met Phe Ile Val Asn Thr Asn Val Pro Arg Ala Ser Val Pro
  1               5                  10                  15

Asp Gly Phe Leu Ser Glu Leu Thr Gln Gln Leu Ala Gln Ala Thr Gly
             20                  25                  30

Lys Pro Pro Gln Tyr Ile Ala Val His Val Val Pro Asp Gln Leu Met
         35                  40                  45

Ala Phe Gly Gly Ser Ser Glu Pro Cys Ala Leu Cys Ser Leu His Ser
     50                  55                  60
```

Ile Gly Lys Ile Gly Gly Ala Gln Asn Arg Ser Tyr Ser Lys Leu Leu
65 70 75 80

Cys Gly Leu Leu Ala Glu Arg Leu Arg Ile Ser Pro Asp Arg Val Tyr
85 90 95

Ile Asn Tyr Tyr Asp Met Asn Ala Ala Ser Val Gly Trp Asn Asn Ser
100 105 110

Thr Phe Ala
115

<210> 5
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<212> PRT
<213> Homo sapiens

<400> 5
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Ala Leu Thr Asn Tyr Gly Phe Tyr Gly Cys Tyr Cys Gly Trp Gly Gly
20 25 30

Arg Gly Thr Pro Lys Asp Gly Thr Asp Trp Cys Cys Trp Ala His Asp
35 40 45

His Cys Tyr Gly Arg Leu Glu Glu Lys Gly Cys Asn Ile Arg Thr Gln
50 55 60

Ser Tyr Lys Tyr Arg Phe Ala Trp Gly Val Val Thr Cys Glu Pro Gly
65 70 75 80

Pro Phe Cys His Val Asn Leu Cys Ala Cys Asp Arg Lys Leu Val Tyr
85 90 95

Cys Leu Lys Arg Asn Leu Arg Ser Tyr Asn Pro Gln Tyr Gln Tyr Phe
100 105 110

Pro Asn Ile Leu Cys Ser
115

<210> 6
<211> 10
<212> PRT

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:synthetic

<400> 6
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1 5 10

Synthesis of Phospholipase A2 Group 5 by Solid Phase Chemical Ligations in the C- to N-Terminal Direction

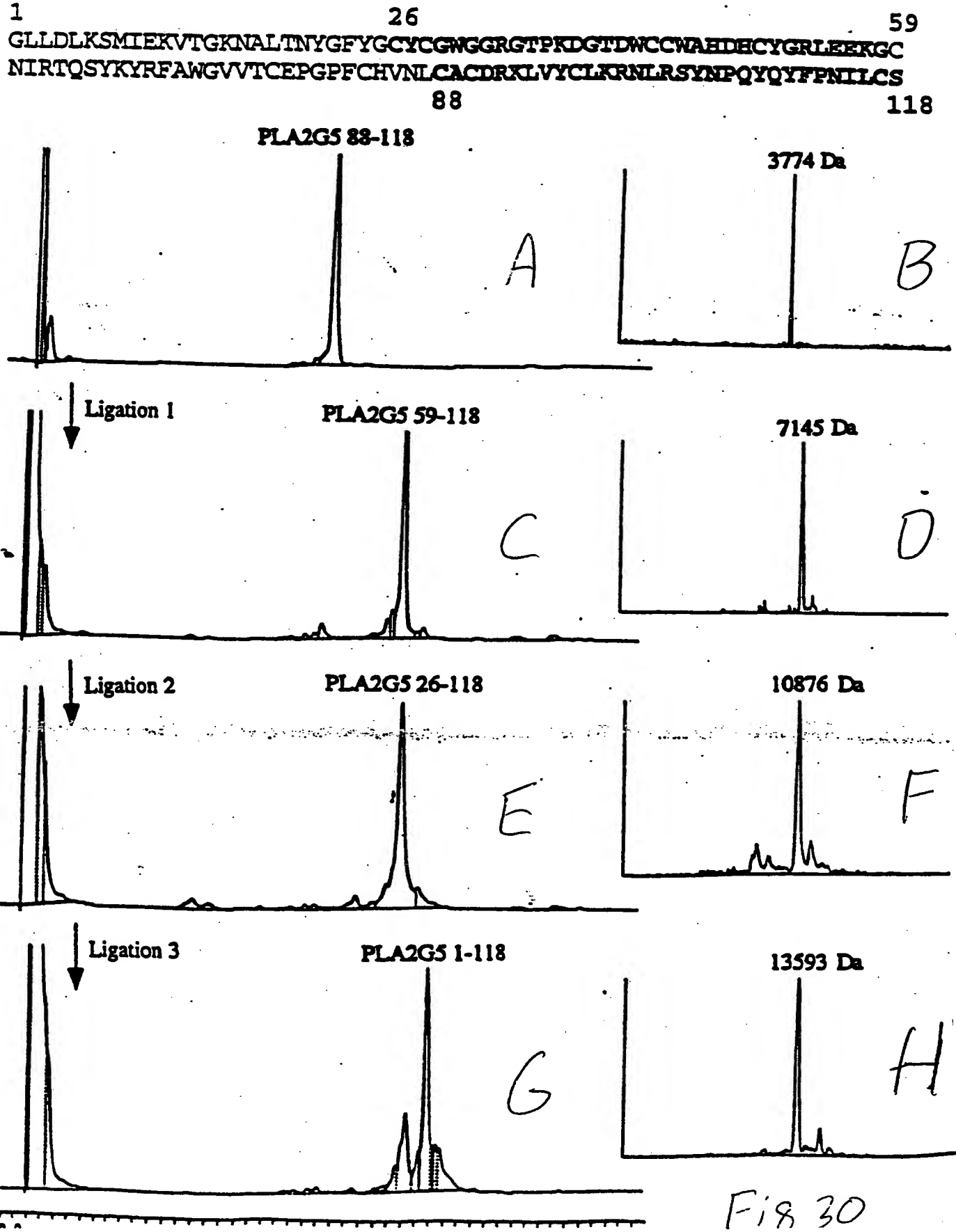

Fig 30

SOLID PHASE NATIVE CHEMICAL LIGATION OF UNPROTECTED OR N-TERMINAL CYSTEINE PROTECTED PEPTIDES IN AQUEOUS SOLUTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in part of, and claims the benefit of, U.S. Provisional Application No. 60/049,553, filed June 13, 1997, herein incorporated by reference, and is a divisional application of, and claims the benefit of, U.S. Patent Application Serial No. 09/097,094, herein incorporated by reference.

Background

Existing methods for, the chemical synthesis of proteins include stepwise solid phase synthesis, and fragment condensation either in solution or on solid phase. The classic stepwise solid phase synthesis of Merrifield involves covalently linking an amino acid corresponding to the carboxy-terminal amino acid of the desired peptide chain to a solid support and extending the polypeptide chain toward the amino end by stepwise coupling of activated amino acid derivatives having activated carboxyl groups. After completion of the assembly of the fully protected solid phase bound peptide chain, the peptide-solid phase covalent attachment is cleaved by suitable chemistry and the protecting groups removed to give the product polypeptide.

Some disadvantages of the stepwise solid phase synthesis method include: incomplete reaction at the coupling and deprotection steps in each cycle results in formation of solid-phase bound by products. Similarly, side reactions due to imperfections in the chemistry, and or impurities present in the reagents/protected amino acids, all lead to a multiplicity of solid phase bound products at each step of the chain assembly and to the formation of complex product mixtures in the final product. Thus, the longer the peptide chain, the more challenging it is to obtain high-purity well-defined products. Due to the production of complex mixtures, the stepwise solid phase synthesis approach has size limitations. In general, well-defined polypeptides of 100 amino acid residues or more are not routinely prepared via stepwise solid phase synthesis. Synthesis of proteins and large polypeptides by this route is a time-consuming and laborious task.

group of the N-terminal cysteine. Steps 2 and 3 can be repeated, as indicated by the arrow marked 4, for additional peptide segments. Also, a cleavable linker for purposes of monitoring the coupling and ligating reactions can be added between the "handle" and the "resin."

FIG. 22 is a reaction scheme for solid phase sequential ligation in the C- to N-terminal direction of PLA2G5.

FIG. 23 is a reaction scheme for synthesizing a Cam ester derivative for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 24 is a reaction scheme for synthesizing the C-terminal peptide segment for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 25A, B, and **C** is a diagram of a scheme for synthesizing an assembled polypeptide via bidirectional solid phase sequential ligation of two or more peptide segments.

FIG 26 are HPLC chromatographs following the solid phase solid phase native chemical ligation of 3 peptide segments in the N- to C- terminal direction, resulting in the assembled peptide, C5a 1-74.

FIG. 27 is a reaction scheme for synthesis of a C-terminal peptide segment for use in the solid phase native chemical ligations described herein, using a CAM ester cleavable handle to remove the synthesized peptide segment from the solid phase.

FIG. 28 are HPLC chromatographs and reconstructed ESI MS of the assembled peptide resulting from solid phase sequential ligation of 3 peptide segments: peptide segment 1 (CADRKNILA) (amino acids 19-27; SEQ ID NO:1), peptide segment 2 (CYGRLEEKG) (amino acids 10-18; SEQ ID NO:1) and peptide segment 3 (ALTKYGFYG) (amino acids 1-9; SEQ ID NO:1) on solid phase in the C- to N-terminal direction, using Fmoc protecting groups.

FIG. 29 are an HPLC chromatograph and ESI MS of the final ligation product, i.e. the first ligation product ligated to the third peptide segment (ALTKYGFYG) (amino acids 1-9; SEQ ID NO:1), resulting from solid phase sequential ligation of 3 peptide segments in the C- to N-terminal direction, using ACM as the protecting group.

FIG. 30A-H are HPLC chromatographs and reconstructed ESI MS of the steps of synthesizing Phospholipase A2 Group 5, a 118 residue protein, using solid phase sequential native chemical ligation of four peptide segments in the C- to N-terminal direction. The first peptide segment is PLA2G5 88-118; the second is PLA2G5 59-87, the third is PLA2G5 26-58,

and the fourth is PLA2G5 1-25. **FIG 30A and B** are an HPLC chromatograph and reconstructed ESI MS of the first peptide segment, respectively. **FIG 30C and D** are an HPLC chromatograph and reconstructed ESI MS, respectively, of the ligation product of the first and second peptide segments (PLA2G5 59-118). **FIG 30E and F** are an an HPLC chromatograph and reconstructed ESI MS, respectively, of PLA2G5 26-118, the ligation product of PLA2G5 59-118 and PLA2G5 26-58 (the third peptide segment). **FIG 30G and H** are HPLC chromatograph and reconstructed ESI MS, respectively, ofPLA2G5 1-118, the assembled polypeptide.

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Aqueous solution: solutions containing water, including up to 8M urea in water, up to 6M guanidine- HCl in water, up to 60% acetonitrile in water.

Assembled Peptide: the final product of a solid phase sequential or bidirectional ligation, after cleavage of the cleavable handle, The assembled peptide comprises at least two separate peptide segments sequentially ligated on a solid phase. The assembled peptide mayor may not have biological activity.

Cleavable Handle: A cleavable moiety that is capable of being selectively cleaved to release the assembled peptide from the solid phase. The cleavable handle must be capable of resisting cleavage under conditions suitable for coupling, activating, deprotecting, ligating, washing, and other steps involved in the formation of an assembled peptide. The cleavable handle must also be stable to conditions used to produce the first peptide segment that is capable of being bound to a solid phase, including, for example, stepwise solid phase peptide synthesis. The cleavable handle preferably is located directly adjacent to the first peptide segment such that upon cleavage of the cleavable handle, the desired assembled peptide is released from the solid phase. The cleavable handle may be selected from any of the variety of cleavable handles used by those in the field. See, e.g., L. Canne et al., Tetrahedron Letters, 38(19):3361-3364 (1997); Ball et al., J. Pept. Sci, 1:288-294 (1995); Funakoshi et al, PNAS USA, 88:6981-6985 (1991);

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+ *Resin-PCL-ONH2*

↓ **1.** pH 4.6, 6M GuHCl, 0.1 M acetate

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS) (1)

↓ **2.** pH 4.6, 6M GuHCl, 0.1 M acetate, 50 mM BrAcOH

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-*COSAc* (1)

↓ + *H*-**CGFRVREFGDNTA**-COS) (2)

3. pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol

Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

↓ **CGFRVREF-GDNTA**-COS) (1+2)

4. pH 4.6, 6M GuHCl, 0.1M acetate, 50mM BrAcOH

Resin- *PCL-oxime- MSC* -LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

CGFRVREF-GDNTA-*COSAc* (1+2)

+ *H*-CADPSEEWVQKYVSDLELSA-*OH* (3)

↓ **5.** pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol

Resin- *PCL-oxime- MSC* –LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG

CGFRVREF-GDNTACADPSEEWVQKYVSDLELSA-*OH* (1+2+3)

↓ **6.** pH 14, 6M GuHCl, 0.1M phosphate, 200mM hydrazine

H-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG**CGFRVREF-**

GDNTACADPSEEWVQKYVSDLELSA-*OH* (1+2+3) (SEQ ID NO:2)

PCL= photocleavable linker

reaction mixture is left standing at room temperature overnight. The next morning, the resin is washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample of resin are removed for monitoring by MALDI MS analysis.

The assembled peptide is removed from the solid phase via base cleavage of the cleavable handle from the remaining resin as outlined above only on a larger scale followed by purification by HPLC or desalting on PD-10 column and lyophilization.

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This example describes solid phase sequential native chemical ligation in the N- to C-terminal direction of C5a, Complement Factor 5A. The sequence of C5a is:
TLQKKIEEEIAAKYKJSVVKKCCYDGACVNNDETCEQRAARISLGPKCIKAFTECCVVAS QLRANISHKDMQLGR (SEQ ID NO:3).

This peptide is prepared using solid phase sequential native ligation of 3 peptide segments: C5a(I-20), C5a(21-46), and C5a(47-74). The procedures used to synthesize C5a by solid phase ligations are identical to those described in the solid phase sequential native ligation of MIF (See Example 5).

Example 5: Solid Phase Sequential Native Chemical Ligation of MIF(1-115) (115 aa) in the N- Terminal to C- Terminal Direction.

The sequence of MIF(1-115) is:
MPMFIVNTNVPRASVPDGFLSELTQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCAL CSLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAASVGWNNSTFA (SEQ ID NO:4)
This peptide is prepared using solid phase sequential native ligation of 3 peptide segments: MIF(1-59) (amino acid 1-59, SEQ ID NO:4) MIF(60-80) (amino acid 60-80, SEQ ID NO:4) and MIF(81-115) (amino acid 81-115, SEQ ID NO:4). **See FIG. 16-20.**

Example 6: Solid Phase Native Chemical. Ligation of Phospholipase A2, group 5(1-118) (118aa) in the C- to N-terminal Direction.

The sequence of Phospholipase A2, group 5 (PLA2G5) is:
GLLDLKSMIEKVTGKNALTNYGFYGCYCGWGGRGTPKDGTDWCCWAHDHCYGRLEE
KGCNIRTQSYKYRFAWGVVTCEPGPFCHVNLCACDRKLVYCLKRNLRSYNPQYQYFPN
ILCS (SEQ ID NO:5).

This peptide is prepared using solid phase sequential native ligation of 4 peptide segments: PLA2G5 (1-25), PLA2G5 (26-58), PLA2G5(59-87) and PLA2G5 (88-118). The procedures used to synthesize PLA2G5 by solid phase ligations are identical to those used for synthesizing the random sequence using ACM protection of the N-terminal Cys residues of the middle segments, as described in Example 9. **See FIG. 22** for the reaction scheme. The Cam ester derivative is synthesized and incorporated into the C-terminal peptide segment according to the diagrams in **FIG. 23, 24/FIG. 27**. The assembled polypeptide, PLA2G5 (1-118), was folded and assayed for biological activity. It had the full activity of a recombinantly expressed PLA2G5.

Example 7: Preparation of Modified C-terminal Peptide Segment (on-resin CAM linker synthesis) (FIG. 27)

The commerical resin of choice (MBHA, any Boc-AA-OCH2-Pam resin) is swelled in DMF
-TFA (1 min x 2) (not necessary if working with MBHA resin)
-DMF flow wash (30 sec x 2)
-addition of activated Boc-Lys(Fmoc)-OH (HBTU/DIEA activation), check for completion of reaction after 10-15 minutes by ninhydrin test
-DMF flow wash (30 sec x 2)
-TFA (1 min x 2) --

Example 8: Solid Phase Native Chemical Ligation of Random Peptide Segments in the C- to N-terminal Direction using Fmoc protection (See FIG. 28)

The following procedures can be used for solid phase ligations in the C- to N-terminal direction, as diagramed in Table 2. By example, a random peptide of: ALTKYGFYGCYGRLEEKGCADRKNILA (SEQ ID NO:1) can be ligated in three peptide segments (from C- to N-terminal direction): segment 1= CADRKNILA (amino acids 19-27; SEQ ID NO:1); segment 2 = CYGRLEEKG (amino acids 10-18; SEQ ID NO:1); and segment 3 = ALTKYGFYG (amino acids 1-9; SEQ ID NO:1).

The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. The modified C-terminal peptide segment (first peptide segment) is dissolved in 6MGu•HCL, 0.1M Na Acetate, pH 4.6 (5 mM first peptide segment) and added to the resin and is left standing at room temperature overnight. The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample is removed for base cleavage and is treated with 8M urea, 0.1M NaPi, pH 7, treated for 2 minutes with 0.25N NaOH in the same 8M urea buffer (resulting pH~14), washed with an equal amount of 0.25N HCl in the same 8M urea buffer (resulting pH~2), and the combined eluants treated with TCEP prior to injection on HPLC.

In preparation for addition of the next segment, the resin is washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5) and drained. The second peptide segment (Fmoc-Cys-peptide-COSR) is dissolved in 6M Gu•HCl, 0.1M NaPi, pH 7.0, 0.5% thiophenol (to at least 10 mM to 50 mM second peptide segment) and added to the resin. The mixture is left standing at room temperature overnight. The resin is washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5), water (1 ml x 5), DMF (1 ml x 5), and the Fmoc protecting group removed by treating with two aliquots of 20% piperidine in DMF (5 min each). The resin is then washed with DMF (1 ml x 5), water (1

Table 2

Polymer-Supported Ligations

C- to N- Terminal Direction

Fmoc Protection

H-CADRKNILA-*CAM-Lys(Levulinic acid)-NH2* (1)

+ Resin- ONH_2

↓ **1.** pH 4.6, 6M Gu•HCl, 0.1 acetate

H -CADRKNILA -*CAM- Lys-oxime*-Resin (1)

+ *Fmoc*-**CYGRLEEKG**-*COSR* (2)

↓ **2.** pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

Fmoc-**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

↓ **3.** 20% piperidine/DMF

H-**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

+ *H*-ALTKYGFYG-*COSR* (3)

↓ **4.** pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-ALTKYGFYG**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2+3)

↓ **5.** pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYG**CYGRLEEKG**CADRKNILA-*OH* (SEQ ID NO:1)

Polymer-Supported Ligations

C- to N- Terminal Direction

ACM Protection

H-CADRKNILA-*CAM-Lys(Levulinic acid)-NH2* (1)

+ Resin- ONH_2

↓ 1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H -CADRKNILA -*CAM-Lys-oxime*-Resin (1)

+ *H*-**C***(ACM)***YGRLEEKG**-*COSR* (2)

↓ 2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-**C***(ACM)***YGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

↓ 3. a. mercury(II)acetate in 3% Aq. AcOH
b. 20% mercaptoethanol in pH 7.5, 6M Gu•HCl, 0.1 M phosphate

H-**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2)

+ *H*-ALTKYGFYG-*COSR* (3)

↓ 4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H- ALTKYGFYG**CYGRLEEKG**CADRKNILA-*CAM-Lys-oxime-Resin* (1+2+3)

↓ 5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYG**CYGRLEEKG**CADRKNILA-*OH* (SEQ ID NO:1)

SEQUENCE LISTING

<110> Canne, Lynne
Kent, Stephen B.H.
Simon, Reyna

<120> Solid Phase Native Chemical Ligation of Unprotected or N-Terminal Cysteine Protected Peptides in Aqueous Solution

<130> GRFN-023/01US

<140> 09/097,094
<141> 1998-06-12

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Lys His Gly Cys Gly Phe Arg Val Arg Glu Phe Gly Asp Asn Thr Ala
35 40 45

Cys Ala Asp Pro Ser Glu Glu Trp Val Gln Lys Tyr Val Ser Asp Leu
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Glu Leu Ser Ala
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Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu Arg Ala Asn Ile
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Ser His Lys Asp Met Gln Leu Gly Arg
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20 25 30

Lys Pro Pro Gln Tyr Ile Ala Val His Val Val Pro Asp Gln Leu Met
35 40 45

Ala Phe Gly Gly Ser Ser Glu Pro Cys Ala Leu Cys Ser Leu His Ser
50 55 60

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Ile Gly Lys Ile Gly Gly Ala Gln Asn Arg Ser Tyr Ser Lys Leu Leu
 65                  70                  75                  80

Cys Gly Leu Leu Ala Glu Arg Leu Arg Ile Ser Pro Asp Arg Val Tyr
                 85                  90                  95

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             20                  25                  30

Arg Gly Thr Pro Lys Asp Gly Thr Asp Trp Cys Cys Trp Ala His Asp
         35                  40                  45

His Cys Tyr Gly Arg Leu Glu Glu Lys Gly Cys Asn Ile Arg Thr Gln
     50                  55                  60

Ser Tyr Lys Tyr Arg Phe Ala Trp Gly Val Val Thr Cys Glu Pro Gly
 65                  70                  75                  80

Pro Phe Cys His Val Asn Leu Cys Ala Cys Asp Arg Lys Leu Val Tyr
                 85                  90                  95

Cys Leu Lys Arg Asn Leu Arg Ser Tyr Asn Pro Gln Tyr Gln Tyr Phe
            100                 105                 110

Pro Asn Ile Leu Cys Ser
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